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Dengue Fever Virus and Japanese Encephalitis Virus Synthetic Peptides, with Motifs to Fit HLA Class I Haplotypes Prevalent in Human Populations in Endemic Regions, Can Be Used for Application to Skin Langerhans Cells to Prime Antiviral CD8$^+$ Cytotoxic T Cells (CTLs)—A Novel Approach to the Protection of Humans

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Abstract. Flaviviruses were reported to induce CD8$^-$ cytotoxic T cells in infected individuals, indicating that nonapeptides, proteolytic cleavage products of the viral precursor protein, enter the endoplasmic reticulum in infected cells and interact with HLA class I molecules. The assembled HLA class I molecules are transported to the plasma membrane and prime CD8$^-$ T cells. Current knowledge of the interaction of viral peptides with HLA molecules is reviewed. Based on this review, an idea is presented to use synthetic flavivirus peptides with an amino acid motif to fit with the HLA class I peptide binding group of HLA haplotypes prevalent in a given population in an endemic area. These synthetic viral peptides may be introduced into the human skin using a lotion containing the peptides ("Peplotion") together with substances capable of enhancing the penetration of these peptides into the skin to reach Langerhans cells. The peptide-treated Langerhans cells, professional antigen-presenting cells, may bind the synthetic viral peptides by their HLA class I peptide-binding grooves. Antigens carrying Langerhans cells are able to migrate and induce the cellular immune response in the lymph nodes. This approach to the priming of antiviral CD8$^-$ cytotoxic T cells may provide cellular immune protection from flavivirus infection without inducing the humoral immune response, which can lead to the shock syndrome in Dengue fever patients. To be able to develop anti-Dengue virus synthetic peptides for populations with different HLA class I haplotypes, it is necessary to develop computational studies to design HLA class I Dengue virus synthetic peptides with motifs to fit the HLA haplotypes of the population living in an endemic region for Dengue fever. Experiments to study Dengue virus and Japanese encephalitis peptides vaccines and their effectiveness in protection against Dengue fever and Japanese encephalitis are needed. The development of human antiviral vaccines for application of viral peptides in a lotion to human skin ("Peplotion") may be useful and affordable for populations of developing countries.

Key words: Dengue fever virus, Japanese encephalitis virus, flaviviruses, synthetic peptides, HLA class I, skin Langerhans cells, CD8$^-$ cytotoxic T cells

Introduction

Porterfield (1) analyzed the phenomenon of antibody-dependent enhancement of viral infectivity and noted that both neutralizing and non-neutralizing antiviral antibodies are synthesized in virus-infected individuals. The latter antibodies might potentiate or "enhance" viral infec-

tivity. Indeed, the phenomenon of immune enhancement of viral infections of flaviviruses (2,3) and human immunodeficiency virus (HIV-1) (4) make effective vaccine development for these diseases all the more difficult. Porterfield (1) indicated that the phenomenon was first described by Hawkes (5), and Hawkes and Lafferty (6) for Murray Valley encephalitis virus (MVEV). Halstead et al. (7) reported on the enhancement of Dengue fever virus infection in macrophages in vitro, in humans with Dengue fever, and in experimental infections of monkeys with Dengue fever virus (1,8). It was noted in the studies by Halstead et al. that infection by any Dengue virus serotype is followed by homotypic immunity but the individual is still fully susceptible to infection with a different Dengue virus serotype. While classical Dengue fever is a benign febrile illness with a characteristic rash, Dengue hemorrhagic fever (DHF) of Dengue shock syndrome (DSS) occurred almost exclusively in children who were experiencing secondary Dengue infection and already had antibodies against one Dengue serotype (9). This phenomenon of enhancement of the virus disease by antiviral antibodies made the development of a vaccine to protect humans an enigma.

To deal with the question of the development of an effective antiviral protective immune response against Dengue fever virus, it is necessary to obtain insight into the nature of Dengue virus infection of cells, how the viral proteins synthesized in the infected cells are processed by the infected cells, and which viral peptides are presented in association with the HLA class I and class II molecules to prime cytotoxic T cells and T helper cells, respectively. Recently, understanding of the organization of the peptide binding grooves of HLA class I and class II molecules, as well as the motifs in the self and viral peptides associated with such peptide binding grooves, was achieved. Knowledge of the intracellular proteolytic processing of structural and nonstructural viral proteins and the interaction of the viral peptides with HLA class I and class II molecules may provide a logical approach to the utilization of professional antigen-presenting cells, like dendritic cells (DC), to present, via HLA class I molecules, a selected repertoire of Dengue virus synthetic peptides with motifs fitting HLA haplotypes of the human populations in endemic regions. Such viral peptides may prime anti-Dengue virus memory $CD8^+$ cytotoxic T cells known to eliminate infected cells before infectious virus progeny can be released from the cells.

The present analysis will analyze the available information on the mode of replication of flaviviruses in the light of the current knowledge on the presentation of peptides by MHC class I and class II molecules by infected cells and professional antigen-presenting cells. The possibility of designing synthetic peptides with motifs to fit different HLA class I haplotypes and the priming of $CD8^+$ cytotoxic T cells will be discussed. The idea that such peptides applied to human skin will penetrate into the skin and stimulate the skin antigen-presenting Langerhans cells to migrate to the lymph nodes to prime CTLs will be evaluated.

Uptake of Flaviviruses by Infected Cells and Intracellular Sites of Virus Replication and Assembly

Flaviviruses interact with cells by two mechanisms: a) virions interact with cellular receptors and enter the cytoplasm by receptor mediated endocytosis and b) by direct fusion with the cell membrane (10). The flavivirions that enter the cells by the acidic endocytotic vesicles are uncoated by the proteolytic enzymes in the endosomes. Treatment of the infected cells with weak bases inhibits flavivirus replication in certain cells and causes the accumulation of virions in endosomes (11). The acidic endosomes contain only three structural proteins: the capsid (C), the matrix (M), and the envelope (E) protein, which undergoes a conformational change. The proteolytic processing of the viral proteins by proteinases in the endosomal vesicles takes place at pH 5.0–6.0, while the vesicle is transported inside the cell cytoplasm. Cathepsin D is responsible for proteolytic cleavage (12). Cathepsin D cleaves peptide bond flanked by F-F, F-Y, and L-F, while cathepsin L cleaves peptide bonds F-R and F-F (13). Thus, endosomal cleavage of the viral proteins will provide a specific set of viral peptides from the C, M, and E viral proteins according to the amino acid sequence of the viral proteins of different flaviviruses.

The flaviviral RNA serves as mRNA (RNA+)

and contains one open reading frame for a polyprotein that is partly inserted into the membrane of the endoplasmic reticulum (ER). Coia et al. (14) suggested that when the N terminus of the polyproteins is in the cytosol, the preM, M, and NS2 respective proteins are inserted through the ER membrane into the ER lumen, and the rest of the polyproteins, including NS2A, NS2B, NS3, ns4a, NS4b, and NS5 at the carboxyterminus, are present in the cytosol. The N-terminal domain of the nonstructural protein of yellow fever virus was reported to be a serine protease responsible for site-specific cleavages in the viral polyprotein (15). The arginine-specific family of serine proteinases cleave after an arginine residue (13), and indeed in yellow fever virus polyprotein the NS2A/NS2B cleavage site is R-S, the NS3/ns4a cleavage site is R-G, the ns4a/NS4b cleavage site is R-V, and NS4b/NS5 cleavage is R-G (16). In contrast, the proteolytic cleavage sites in yellow fever virus polyprotein between C/prM is T-L, prM/M is R-A, M/E is S-A, E/NS1 is A-D, and NS1/N53A is A-V (16).

A similar situation with polyprotein cleavage domains was reported for Kunjin virus polyprotein (14). These authors' suggestion that the prM, M, E, and S1 portion of the Kunjin virus polyprotein is inserted into the lumen of the endoplasmic reticulum is in agreement with the mode of action of the signal proteinases present in the ER lumen. Such enzymes cleave peptide bonds at specific points in the polypeptide adjacent to small amino acid residues such as alanine (A), cysteine (C), glycine (G), and serine (S). This supports the Coia et al. (14) suggestion that the preM, M, E, and NS1 portion of the viral EM polyprotein is processed inside the ER lumen. An additional support for this suggestion is the Buckley et al. (17) experiment in which antibodies to the structural E protein of yellow fever virus and to nonstructural protein NS5 were used to identify the intracellular sites of the viral proteins. The anti-E antibodies detected the protein in the endoplasmic reticulum around the nucleus and in small cytoplasmic vesicles between the ER and the cell membrane, which was also stained. In contrast, the anti-NS5 antibodies detected the viral protein around and inside the nucleus. This viral protein was localized in larger vesicles dispersed in the cytoplasm. Thus, the association of the prM, M, E, and NS1 proteins with the ER while the rest of the viral proteins are inside the cytoplasm may have important implications not only for the formation of mature virions in the infected cells but also for the immune response to the viral proteins.

The vesicles described by Buckley et al. (17) are the transport vesicles that are released from the ER membrane and translocated by a specific mechanism to the pre-Golgi and Golgi apparatus (18). Guirakhoo et al. (11) indicated the proteolytic cleavage and maturation of the prM, M, E, and S1 take place in acid post-Golgi vesicles, and the maturation process can be inhibited by acidotropic amines such as chloroquine, ammonium chloride, or methylamine (19), in agreement with the findings of Buckley et al. (17). Sarasate and Kuismanen (20) reported that pre- and post-Golgi vacuoles operate in the transport of Semliki Forest membrane glycoproteins to the cell surface.

How Viral Proteins are Degraded to Peptides and Inserted into MHC Class I and II Molecules

The membrane of the ER separates the lumen of the ER from the cytosol, generating a compartment in the cytoplasm into which glycoprotein could be inserted and processed under controlled conditions. The ER membrane contains inserted proteins that are actively responsible for transporting specific sets of proteins and peptides in a selective way from the cytoplasm into the ER lumen to fulfill specific cellular functions. Among the specific activities of the ER membrane is the translocation of MHC class I and class II molecules from the cytoplasm and the transfer of peptides that are the cleavage products of cytosolic proteasomes. The ability of flaviviruses to insert their structural proteins into the ER membrane suggests that the virus, being an obligate cellular parasite, is utilizing cellular processes for the formation of its virus progeny.

Cytosolic Proteasomes Near the ER Membrane

Proteasomes are nonlysosomal cytosolic multicatalytic proteinase complexes. These enzymes have an alkaline pH optima and do not require ATP for activity. The proteasomes are complexes of high relative molecular mass (M_r ~ 600 kD), consisting of 2–30 subunits with M_r ranging

15–30 kD. Ortiz-Navarrete et al. (21) reported that at least one of these subunits is encoded by the mouse MHC in the region between the K locus and the MHC class II region. The expression of this gene is inducible by interferon-γ. Martinez and Monaco (22) also reported that the class II region of MHC contains at least two genes that code for two subunits of the proteasome and isolated the cDNA corresponding to one of the LMP genes. Kelly et al. (23) identified the second MHC class II gene that codes for a proteasome subunit.

The proteasomes are cysteine proteinases and can cleave more than one type of peptide bond. They may be classified according to Bond and Butler (13) as trypsinlike (R-X), chemotrypsinlike (F-X), and peptidy-gentamyl peptide bond hydrolyzing enzyme (E-X). The proteasomes, being located in the cytosol, can degrade cellular as well as flaviviral proteins that are cleaved from the viral polyprotein as described above. Proteasome degradation of viral polyproteins that were not inserted into the ER membrane may also occur. The peptides that result from proteasome degradation of cellular and viral proteins concentrate near or at the cytosolic side of the ER membrane.

Transporter Proteins in the ER Membrane Are Coded by Two Genes in the Class II Region in Human and Murine DNA

Deverson et al. (24) identified the MHC class II region that encodes proteins that are related to the multidrug resistance (MDR) family of transmembrane transporters. In human class II regions the genes are located between DP and DQDR (25). These two genes (RING 4 and RING 11) were identified by Kelly et al. (26). These two proteins form a complex in the ER membrane that transports peptides from the cytosol to the ER. Altaya (27) reported that the murine MHC class II region contains two genes (Ham 1 and Ham 2) that encode proteins belonging to a superfamily of ATP-dependent transport proteins that mediate the transport of peptides from the cytosol into the ER lumen. Thus the proteasome-cleaved cellular and viral proteins are transported from the cytosol to the lumen of the ER.

HLA (MHC) Class I and II Glycoproteins Are Translocated by the N Terminus Through and Anchored in the ER Membrane with the N Termini in the ER Lumen Capable of Binding Antigenic Peptides

Two different mechanisms govern the behavior of MHC class I and II glycoproteins in their translocation through the ER membrane and processing in the ER lumen, as well during transport to their ultimate positions on the cell membrane. However, the translocation of the different protein subunits is carried out by the signal recognition complex (28) that recognizes the signal sequence of the protein subunits and translocates the N terminus through the ER membrane by an ATP-consuming process until the membrane anchor domain of the polypeptide subunit is inserted into the ER membrane.

Class I MHC Molecules

MHC Class I Heavy Chain Molecules Assemble with Light Chains (β2 Microglobulin in the Presence of Peptides at 37°C and in their Absence at Lower Temperatures

Studies by Townsend et al. (29) revealed that HLA-restricted cytotoxic T cells (CTLs) are specific for an antigenic epitope in the matrix protein of influenza virus and described the sequence of the peptide from the influenza virus matrix protein that was recognized by the human CTL in association with HLA-A2 molecules. Analysis of a Ranscher leukemia virus transformed C57BL 6 mouse T cell line (designated RMA) mutant cells (RMA-S) revealed that this cell line was defective in assembly of MHC class I molecules at 37°C (30). The defect in RMA-S cells was corrected by gene transfer of a putative peptide transporter (27,31,32). These results and those of Elliot et al. (33) led to the conclusion that specific peptides derived from cellular and viral proteins stabilize or induce in MHC class I heavy chain, which results in an increase of the binding affinity between the heavy chain and the β_2 microglobulin (β_2m) molecule. Elliot et al. (33) reported that 9–10 amino acid (aa) peptides can induce folding of the MHC class I heavy chain

in the absence of β2m. To alter the conformation of free heavy chains, the peptides must be of the correct size. At a lower temperature 19–33°C peptide-less ("empty") MHC class I molecules made of both heavy and light chains are transported to the cell membrane (34).

Structure of Human Class I Histocompatibility Antigens and the Peptide Binding Groove

X-ray crystallography of human HLA-A2 antigen by Bjorkman et al. (35) revealed that the class I histocompatibility antigen from human cell membranes has two structural motifs: The membrane proximal end of the glycoprotein contains two domains with immunoglobulin folds that are paired in a novel manner, and the region distal from the membrane is a platform of eight antiparallel β-strands topped by α-helices to which a "foreign antigen" is bound. The latter was identified to be a peptide (35–37). A refined analysis of the histocompatibility antigen HLA-A2 provided detailed information on the structure and composition of the peptide binding groove (38).

It was found that the conserved amino acids side chains are clustered in the narrower ends of the groove, while the most polymorphic amino acids in the peptide binding domains in mouse and human alleles fill up the central and widest portion of the groove. The peptide binding groove is ~25 Å deep. This suggested to Bjorkman et al. (37) that the bound peptide will make contacts on three of its surfaces: to the bottom and to the two sides of the groove. The binding groove contains a number of tyrosines and many polar or charged residues as well as nonpolar amino acids, which can accommodate an extended polypeptide chain of eight residues. Van Bleek et al. (39) suggested that the clustered changes in the MHC class I peptide binding groove determine the motif of the peptides that are capable of binding to the groove.

Characterization of Self and Viral Peptides in the MHC Class I Peptide Binding Groove

Silver et al. (40) reported the structure of the complex between the human class I histocompatibility glycoprotein HLA-AW68 and the influenza virus nucleoprotein peptide Np91-99 as determined by x-ray cryocrystallography. It was found that residues on both ends of the peptide are substantially buried in the peptide binding groove, while the amino acids in the middle of the peptide are predominantly exposed. This finding is in agreement with Saper et al. (38), who described the presence of six pockets, each accommodating one amino acid of the peptide. Zhang et al. (41) reported that a nuclear capsid protein octa peptide (N52-59) of vesicular stomatitis virus resided in the peptide binding groove in a single and well-ordered β-strand.

Direct analysis and sequencing of peptides that were released from the peptide binding groove of purified HLA-B27 MHC class I molecules provided a motif of the peptides derived from cellular (self) proteins and from viral proteins. The peptides contained nine amino acid residues with the motif (1)RRYQKSTEL(9) for a peptide derived from human histone H3 and a peptide derived from HIV-1 gag (aa265–276) (1)KRWIILGLN(9) (42). Guo et al. (43) reported up to 11 amino acids peptides can be found attached to the peptide binding groove and suggested that the excess amino acids bulge in the middle of the peptide. The motif for the peptides in HLA-AW68 was found for self peptide as well as influenza virus NP91-99 to be (1)KTGGPIYKR(9).

Falk et al. (44) reported the motif of peptides bound to HLA-A2 molecules. The motif was for self peptides conformed for reported viral peptides such as HIV-1 reverse transcriptase aa461–468; HIV-1 gag protein derived peptides aa219–233, aa418–443, and aa446–460; and influenza matrix protein peptide aa57–68 (1)FIL:GFVFTL(9). The peptide binding groove was reported by Schumacher et al. (45) to be able to select and bind the nine amino acid peptides, even when they were a minor component in a mixture of longer peptides. From the above studies it is noted that three motifs were found in the MHC class I bound peptides: a) HLA-B27, in most of the peptides the peptide starts and ends with arginine (R) or lysine (K); b) HLA-AW68, the second amino acid is valine (V) or threonine (T), and the last amino acid is arginine (R) or lysine (K); and c) HLA-A2, the dominant amino acids leucine (L) in position 2 and valine (L) in

position 9. These findings may be taken to indicate that peptides cleaved from cellular or viral proteins by the cytosolic proteasomes (which proteolytically cleave near R or K) are pumped in the lumen of the ER, where they mix with peptides generated from cellular and viral glycoproteins by ER lumen peptidases. The correct-size nine amino acid peptide is selected by the peptide binding groove of MHC class I molecules, which attains a conformation that allows the binding of β2m molecules (at 37°C).

Transport of MHC Class I Molecules with a Peptide Bound to the Cell Membrane

The MHC class I mature molecules are transported by vesicles from the ER through the pre-Golgi and Golgi apparatus, where the glycosylation of the molecules take place before transport and positioning in the cell membrane with the peptide binding groove on the cell surface and the carboxyterminus in the cytoplasm. It was reported that a portion of the ER membrane, presumably containing inserted MHC class I molecules, is released as vesicles that are covered by a protein (18). This protein is β-CoP, a 110 kD protein that associates with vesicles that are not coated with clathrin (46). In addition to the bound form, β-CoP forms a cytosolic complex of M_r 550.000. Immunoelectron microscopy has localized β-CoP to nonclathrin-coated vesicles and cisternae of the Golgi apparatus.

The role of β-CoP in vesicular transport was studied with the ts045 mutant of vesicular stomatitis virus. The viral glycoprotein (VSV-G) in infected Vero cells is blocked in the ER at the nonpermissive temperature of 39.5°C but is transported to the cell surface at 31°C. At 15°C or 20°C VSV-G accumulates in an intermediate compartment. Pre- and post-Golgi vacuoles operate to transport Semliki Forest virus membrane protein (20). Duden et al. (46) suggested that the coat of Golgi-derived vesicles is composed of a set of proteins: α-CoP (160 kD) may be related to clathrin heavy chain, β-CoP (110 kD) may be homologous to β-adaptin, γ-CoP (98 kD) may be homologous to another member of the adaptin family, and δ-CoP (61 kD) may be similar to a 50 kD adaptin subunit.

The vesicles that transport the glycoproteins that were inserted into the ER membrane must fuse, first to form a vesicle, and then they must fuse with the cisternae of the Golgi apparatus, and finally they must correctly fuse with the cell membrane for the MHC class I molecules to properly protrude from the cell membrane. Recent studies by Söllner et al. (47) showed that the targeting of the transport vesicle is achieved by a docking mechanism by which a protein in the vesicle membrane docks to a receptor protein in the next membranous target (18). The vesicles are transported through the cytoplasm to the cell membrane by attachment to the cytoplasmic microtubules, which are also involved in cell motility (48).

How the T Cell Receptor (TCR) Recognizes the Peptide in the Peptide Groove of the MHC Class I Molecules

Shin et al. (49) reported that the α chain of the T cell antigen receptor (TCRA) after synthesis forms a complex after translocation into the ER membrane. However, the putative transmembrane amino acid sequence is incomplete to anchor the polypeptide on its own. The TCR is a disulfide-linked heterodimer of α and β polypeptide chains with polymorphic components of the CD3 molecule. The TCR protein complex is transported from the lumen to the ER to the cell membrane, where they are capable of interaction with the "full" peptide binding groove on MHC class I molecules.

Bjorkman and Davis (50) and Saper et al. (38) indicated that T-cell receptor molecules show structural similarity with the Fab portion of immunoglobulins and the size of the Fab footprint is about 20 × 30 Å, which suggests that TCR could simultaneously recognize a peptide and the α helical edges of the peptide binding groove (about 20 Å wide and 30 Å long). It was suggested that the third and most hypervariable regions of TCR α and β chains might be the primary contact to the peptides in the peptide binding groove (51).

Removal of MHC Class I Molecules from the Cell Membrane

Vega and Strominger (52) reported that HLA class I molecules are removed from the plasma

membrane, in the absence of specific antibody, by endocytosis.

MHC Class II Molecules

MHC Class II α, β, and Invariant Polypeptides

Cresswell et al. (53) studied the structural and functional aspects of the HLA class II glycoproteins α and β, and their association with the invariant (I) polypeptide chain. The class II α and β polypeptide chains are cotranslationally translocated through the ER membrane and are glycosylated to generate a single N-linked glycan on the β subunit and two on the α subunit. The α polypeptide contains 232 amino acids divided into two external domains α1 (aa1–87) and α2 (aa88–181), a connecting peptide (aa182–194), a hydrophobic transmembrane region (aa195–217), and an intracytoplasmic region (aa218–232) (54). The α1 domain and the β1 domain on the β polypeptide are highly polymorphic and appear responsible for binding peptides at what appears to be a single site for recognition by MHC-restricted antigen-specific T cells (55). However, the interaction of peptides with α1 and β1 peptide binding domain of class II molecules is prevented by the rapid association of 31 kD third subunit (designated invariant [I] chain) with the α,β heterodimer. The I chain is not encoded by one of the MHC genes and has no sequence homology with the α and β chains or with the immunoglobulin superfamily. The I polypeptide has 2 N-linked glycans and is inserted into the membrane with its N terminus, which forms a cytoplasmic domain consisting of 30 amino acids.

Transport of Class II Complex to the Cell Membrane

Within 30 min of their association in the ER, the three chains of class II MHC leave the ER and are transported by vesicles that shuttle through the Golgi apparatus, where the N-linked glycans are processed and the 0-linked glycans are added to the I chain. After 1–3 hr proteolysis of the I chain occurs and dissociates from the αβ heterodimer (53). The α1 and β1 domains of the α and β polypeptides are now able to reach the correct conformation, namely, the α1 and β1 N-terminal amino acid sequences form the rim of the peptide binding groove, while the β-sheets of the β molecule form the bottom of the groove (56).

MHC Class II Empty Molecules Interact with Peptides

Riberdy et al. (57) indicated that the cytoplasmic portion (the N-terminus) of the invariant chain contains a cytoplasmic signal that targets the class II invariant chain to an acidic endosome compartment. Thus, one source of peptide that binds to the MHC class II peptide binding groove should be the peptides derived from the degradation of the class I polypeptide, and the other source may be the peptides in the endosomes generated by the proteolytic cleavage of exogenous proteins such as endocytosed virus particles cleaved by the endosomal cathepsins. If viral glycoproteins such as those of flaviviruses are situated in the ER membrane near the class II molecules, then the vesicle transporting the MHC class molecules to the endosomal vacuoles and the Golgi apparatus and the cell membrane will transport the viral structural protein. An indication that events happen in HIV-1 infected cells is the finding that the HIV-1 virion contain MHC class II molecules as well as the viral gp160 glycoprotein (58,59).

Riberdy et al. (57) reported that class II molecules were efficiently loaded with antigenic peptides derived from the invariant chain. Rudensky et al. (60) isolated and sequenced peptides that were removed from the mouse class II molecules and found that most of the isolated peptides from self proteins and murine leukemia virus (MuLV) range 13–16 amino acids per peptide. They identified the amino acids that determine the motif of the peptides, presumably the amino acids in the peptide that interact with side chains of amino acid residues in the α1 and β1 domains of the peptide binding groove. Hunt et al. (61) indicated that between 650 and 2000 different peptides are associated with MHC class II molecules of I-A^d. All the peptides were derived from secretory or membrane proteins. The peptides were 16–18 residues long, had ragged NH_2 and COOH termini, contained a six-residue binding groove, and were suggested to be open at both ends.

The vesicle and the endosome interaction and transport through the Golgi apparatus (details unknown) will eventually reach and fuse with the cell membrane, and the MHC class II molecules with a 16–18aa peptide in the peptide binding groove will appear on the cell membrane.

TCRs of T Helper Cells Interact with MHC Class II Molecules

The different structures of the peptide binding grooves of MHC class I and II are the basis for their selectivity. While class I molecules interact with cytotoxic T cells and prime these cells to recognize the presented peptide, the class II molecules interact with T helper cells, which transfer the information about the antigenic peptide to cells for the synthesis of specific antipeptide antibody.

Thus, the presentation of viral peptides on class I and II molecules depends on their cytoplasmic compartments, which determine the mode of proteolytic cleavage and presentation to class I or II MHC molecules. Nonstructural viral peptides will be proteolytically cleaved by cytosol proteasomes and therefore will generate peptides with MHC class I motifs and cytotoxic T cells. Viral glycoproteins may be cleaved by endosomal proteases, and thus longer peptides capable of interaction with class II molecules will be formed, leading to the synthesis of antiviral antibodies.

Cellular Immune Response to Flaviviruses and the HLA Class I Haplotypes in Populations of Endemic Regions

Flaviviruses Are Able to Stimulate the Immune Response of the Infected Host by Priming Antiviral Cytotoxic T Cells and Antiviral Antibodies

Hill et al. (62) used a panel of recombinant vaccinia viruses that expressed portions of the complete cDNA of Kunjin virus genome to infect target cells. Anti-Kunjin CTLs were generated to determinants derived from nonstructural proteins; NS3, ns4a, and NS4b were particularly dominant in most virus strains. Usually only one class I MHC restriction element was involved. CTLs were obtained to West Nile virus proteins.

Bukowski et al. (63) reported on T-cell responses in mice and humans of both helper and cytotoxic types. A $CD8^-$ CTL response in Dengue 4 virus immune donor lysed Dengue 4 and Dengue 2 virus infected cells and cells that were pulsed with antigen from all four Dengue virus subtypes. Two CTL determinants were reported: one in E and one in NS2B/NS3/ns4a proteins.

The recognition of helper T cell epitopes in the envelope (E) glycoprotein of the flaviviruses Japanese encephalitis, West Nile, and Dengue viruses was reported by Kutubuddin et al. (64). Roehrig et al. (65) used computer-designed synthetic peptides according to the putative antigenic domains in the E glycoprotein of Murray Valley encephalitis virus to elicit antibody response using adjuvants. However, the ability of anti-Dengue fever virus antibodies to enhance the severity of the disease in children previously infected with another Dengue virus type complicates the development of either killed or live attenuated vaccines.

Histocompatibility Antigens in a Human Population in an Endemic Region for Dengue Virus and its Relation to Dengue Hemorrhagic Fever and Dengue Shock Syndrome

Chiewsilp et al. (66) reported in a pilot study on associations between the frequency of HLA-A2, HLA-B blank, and LHA-B13 and Dengue hemorrhagic fever and Dengue shock syndrome. An increased frequency of HLA-A1 and HLA-A9 was found in patients with primary Dengue disease. The finding of increases of HLA-A2 and HLA-B blank and decreases in HLA-B13 only in patients who developed shock was regarded as "provocative", since children who did not develop shock had a similar distribution of HLA types as the controls.

Can Priming of CTLs Against Viruses by Synthetic Peptides be Achieved In Vivo Without Inducing Antiviral Antibodies?

Cytotoxic T cells to respiratory syncytial virus (RSV) nonstructural proteins were reported to effectively eliminate cells infected by the virus

in the absence of a strong humoral response (67). Kast et al. (68) reported that mice immunized with Sendai virus nucleoprotein synthetic peptide aa321–336 by subcutaneous inoculation induced virus-specific CTL memory response.

Schultz et al. (69) reported that the subcutaneous infection of the T-cell epitope, a synthetic unmodified free 15 amino acid peptide of the lymphocytic choriomeningitis virus (LCMV) nucleoprotein, emulsified in incomplete Freund's adjuvant, induced in mice an anti-LCMV specific protective state. This immune response was correlated with the ability of the immunized mice to respond to the peptide challenge by $CD8^+$ virus specific T cells.

Interaction of Viral Peptide Cytoplasmic Degradation Products of Viral Proteins with HLA Class I Molecules Responsible for the Induction of $CD8^+$ CTLs in Humans

The amino acid sequence of viral nonapeptides extracted from antiviral CTLs revealed that different HLA class I haplotypes require different amino acid sequences of the viral nonapeptide to fit their peptide binding grooves, which are changed due to mutations of the HLA class I genes. Jardetsky et al. (42) reported that HIV-1 gag derived nonapeptide [gag aa265–276 (1) KRWIILGLN (9)] was extracted from the peptide binding grooves of purified HLA-B27 protein molecules. However, the HIV-1 gag peptides derived from HLA-A2 protein molecules were nonapeptides with a different amino acid motif (see previous discussion).

These and other studies of the viral peptide motifs in different HLA haplotypes indicated that the viral proteins, in most cases the nonstructural viral proteins, are degraded by the cytoplasmic proteasomes system (see previous discussion) and the peptides are pumped into the endoplasmic reticulum. Of the large number of viral peptides that resulted from the proteolytic degradation of the viral nonstructural peptides, only a few may fit perfectly into the binding groove of the HLA class I proteins molecules. The HLA class I molecule is assembled and folded when the β2-microglobulin molecule interacts with the HLA class I heavy chain, anchored in the membrane of the endoplasmic reticulum, to obtain its correct conformation (see previous discussion).

Another source of the viral peptides that interact with HLA class I molecules in the endoplasmic reticulum are the viral peptides derived from the signal peptides of the viral glycoproteins. The viral signal peptides are proteolytically cleaved after the entry of the viral glycoprotein molecule through the endoplasmic reticular membrane into the lumen. These viral peptides must have the proper amino acid motif to be able to fit the HLA haplotype of the human cells.

Administration of Viral Peptides to Skin Langerhans Cells as a Novel Approach to Priming of Antiviral $CD8^+$ Cytotoxic T cells

In Vivo Site of Peptide Injection to Stimulate Antiviral CTLs

In the study by Schulz et al. (69) cytotoxic T cells to LCMV were induced by the subcutaneous injection of viral synthetic peptide into mouse skin. It is logical to assume that the type of cells in the skin capable of interacting with the viral peptides are the skin Langerhans cells (70). Cherrie et al. (71) reported that human cytotoxic T cells stimulated by antigen on dendritic cells recognized respiratory syncytial virus proteins. Kripke et al. (72) provided evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. McKinney and Streilein (73) demonstrated that sensitization enhances the capacity of Langerhans cells to prime cytotoxic T cells in vivo. The expression of HLA class I molecules on the plasma membrane of Langerhans cells (70) and the ability of Langerhans cells loaded with antigen to travel from the epidermis, through the lymphatics of the dermis, to the lymph nodes, the site where they perform antigen presentation to $CD8^+$ or $CD4^+$ T cells, makes these cells of marked importance for the priming of the cellular antiviral immune response.

Priming of $CD8^+$ Cytotoxic T Cells by Synthetic Peptides Under In Vitro Conditions

Skin Langerhans cells belong to the dendritic cell lineage that originate in the bone marrow from

specific progenitor cells. Dendritic cells, enriched from human peripheral blood, were exposed to HIV in vitro and small numbers of these cells were added to T lymphocytes. After 6 days cytotoxic T cells were obtained that killed infected autologous but not allogeneic phytohemagglutinin stimulated blast cells. The primed cytotoxic T cells showed cell killing was restricted by HLA class I. Dendritic cells pulsed with a HIV gp 120 peptide (aa111–126) stimulated cytotoxic T cells under in vitro conditions. The report on the priming of $CD8^-$ T cells in vitro by a synthetic viral peptide presented by dendritic cells can be used as a method to evaluate the priming capacity of the synthetic viral peptide (74).

Computer Analysis of the Cleavage Pattern of Viral Structural and Nonstructural Proteins and Computational Determination of Viral Peptide Motifs

Computer analysis of the sequence of proteolytic cleavage of viral proteins can be used to select the amino acid sequences in viral proteins with motifs to fit HLA class I haplotypes. Computer analysis is based on the proteolytic cleavage pattern of the cytosolic proteasome proteolytic enzymes and the signal peptidases in the endoplasmic reticulum (Y. Becker, to be published). In conjunction with computer analysis of the cytoplasmic cleavage pattern of the viral proteins, it is necessary to use a computational analysis of a different type to determine the three-dimensional properties of the peptide binding grooves of HLA haplotypes, for which x-ray crystallographic analyses are not available. Sezerman et al. (75) reported on the computational determination of peptide-HLA three-dimensional structure based on the coordinates of known HLA haplotypes. Rosenfeld et al. (76) developed a successful computation analysis of the structure of peptides bound to MHC class I. The use of these computational technologies will provide new tools to predict the amino acid motifs of viral peptides capable of interaction with and presentation by HLA haplotypes prevalent in human populations in endemic areas to protect against virus infections.

How to Achieve Priming of Anti-Dengue Virus $CD8^+$ Cytotoxic T Cells to Protect Against Dengue Fever

Design of Specific Dengue Viral Peptides to Fit HLA Haplotype of Human Populations in Endemic Areas

To determine the properties of Dengue virus or Japanese encephalitis virus peptides that are involved in priming of $CD8^-$ cytotoxic T cells, it is possible to extract peptides from white blood cells from the peripheral blood of Dengue virus infected individuals and to determine the amino acid sequences of the isolated peptides. These amino acid sequences will be compared to the amino acid sequences of Dengue virus proteins. In addition to this study, it may be possible to use the computational approach to predict the motifs of the Dengue virus peptides, which may be used to immunize human populations with different HLA haplotypes to prime CTLs. The in vitro priming by the viral peptide treated dendritic cells of $CD8^-$ cytotoxic T cells capable of killing Dengue virus infected cells may provide the basis for immunization of humans with Dengue virus synthetic peptides. A similar approach can be used in the design of Japanese encephalitis peptides for the priming of CTLs.

Immunization of Humans with Synthetic Dengue Virus Peptides Introduced by a "Peplotion" into the Skin of Humans to Stimulate Langerhans Cells to Prime Cytotoxic T Cells

The idea that is presented in the present analysis is that synthetic Dengue virus peptides should be identical to Dengue virus peptides that are naturally presented by antigen-presenting cells to cytotoxic T cells in infected individuals. However, since Dengue virus endemic areas are inhabited by human populations that vary in their HLA class I haplotypes, it is necessary to assume that different Dengue virus peptides may induce cytotoxic T cells. The computational approach to three-dimensional analysis of HLA class I haplotypes (75) and the motifs of the peptides (76) may enable the design of Dengue virus

synthetic peptides to fit the grooves of the different class I haplotypes. It is necessary, therefore, to determine the HLA class I haplotypes prevalent in the different human populations of Dengue virus endemic areas around the world.

The synthetic viral peptides should be emulsified in a skin lotion (designated "Peplotion") that can be applied to the skin of each individual in the human population in the Dengue virus endemic region. Such applications of the "Peplotion" to the skin can be done by the members of the population without need for syringes or medical or paramedical staff. The "Peplotion" containing Dengue virus peptides may not need refrigeration and thus are useful in hot climates. The "Peplotion" Dengue virus peptide vaccine may contain, in addition to the HLA class I virus peptide(s), substances that will enhance the penetration of the peptide molecules through the epidermis to the layer of the Langerhans cells. In addition, the "Peplotion" may contain a mosquito repellant. It is hoped that the Dengue virus "Peplotion" may be capable of inducing cytotoxic T cells to Dengue virus that will be able to destroy Dengue virus infected cells in Dengue virus infected individuals. Elimination of the Dengue virus infected cells prior to release of the virus progeny will prevent the Dengue fever and its outcome, the shock syndrome. The use of the Dengue virus "Peplotion" will prevent or markedly reduce the synthesis of anti-Dengue virus neutralizing antibodies, which were implicated in the development of the Dengue shock syndrome in Dengue fever patients (3).

Synthetic Dengue virus peptide epitopes, capable of interaction with the peptide binding grooves on HLA class I molecules, due to their resemblance to viral peptides presented by HLA class I on infected cells when introduced to human skin Langerhans cells, will be presented in the lymph nodes to $CD8^-$ T cells to prime CTLs. Introduction of Dengue virus synthetic viral peptides with the amino acid motifs to fit the HLA class I peptide grooves on the HLA class I haplotypes prevalent in human populations of endemic regions where highly pathogenic viruses prevail (for which conventional vaccines cannot be developed) can be used to prime antiviral cytotoxic $CD8^-$ T cells. Since human ethnic populations differ in their HLA class I haplotypes, the viral synthetic peptides with HLA class I motifs to be used as "Peplotions" must be made to fit the prevalent HLA haplotypes in each human population to be immunized. Such a "Peplotion" vaccine will resist climatic conditions prevailing in Dengue fever endemic regions, can be easily applied to the skin without the need of syringes and medical personnel, and can be affordable to populations in developing countries.

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